Virus Genes 20:1, 11–17, 2000

Identification of a Novel Plant Virus Promoter using a Potyvirus Infectious Clone

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Received April 18, 1999; Accepted June 22, 1999

Abstract. A putative promoter from the strawberry vein banding caulimovirus (SVBV) genome was identified by its ability to drive infection with full-length cDNA of the zucchini yellow mosaic RNA potyvirus (ZYMV). A high rate of infection was obtained with the cDNA under control of the SVBV promoter using particle bombardment technology. The SVBV promoter shows 60% homology to the cauliflower mosaic virus 35S promoter in the domain spanning the conserved motifs of CCACT (at −83) and the TATA box (at −31), to the transcription start. The 3′-end one-third of the putative promoter (328 bp) was sufficient to invoke full infectivity with the ZYMV clone, and drove transient reporter gene expression in *Solanaceae* and *Cucurbitaceae* transformed with a binary plant transformation vector. Stable expression of a reporter gene (GUS) under control of the truncated SVBV promoter was shown in transformed tobacco shoots in roots, leaves and stems.

Key words: plant viral promoter, caulimovirus, strawberry vein banding virus, zucchini yellow mosaic virus, particle bombardment

Introduction

In vivo infection with cDNA of several different plant RNA viruses under control of the cauliflower mosaic virus (CaMV) 35S promoter has been previously described (1–9). The full-length cDNA (FLC) of zucchini yellow mosaic virus (ZYMV) was constructed under control of a bacterial promoter (10), and subsequently under control of the 35S CaMV promoter (6). Very efficient infection was achieved by particle bombardment of host plants with the FLC of ZYMV driven by the CaMV 35S promoter (6,11). This method is based on the delivery of metal particles coated with cDNA of a virus into the plant cell, which is then transcribed by an endogenous plant polymerase possessing affinity for the promoter located upstream of the viral cDNA.

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Several transcriptional promoters have been identified in members of the caulimovirus subgroup of the pararetrovirus supergroup, including CaMV (12), soybean chlorotic mottle virus (13), figwort mosaic virus (FMV) (14), cassava vein mosaic virus (CVMV) (15,16), and peanut chlorotic streak caulimovirus (17). The CaMV 35S promoter was the first characterized (12,18–21) of the sequenced viral promoters of transcription. Additionally, another active transcriptional promoter was identified in the CaMV genome, the 19S promoter (22,23). The 35S CaMV promoter is extensively used in the expression of transgenes due to its strong constitutive activity and broad range of application in both monocotyledonous and dicotyledonous plant species (24). The CaMV 35S promoter harbors two essential motifs universal to RNA polymerase II transcription initiation regions: a TATA box (−31 from the transcription start site), and a CCACT box (−86 from the transcription start site) (23).

Strawberry vein banding virus (SVBV) is a circular double-stranded DNA virus, a member of *Caulimoviridae*, with a genome size of 7876 bp (25), encoding seven open reading frames. Potential TATA boxes were found in several positions in the SVBV genome (25). The genome organization of SVBV is similar to that of CaMV (25), and its amino acid sequence shows homology to that of CaMV, FMV and carnation etched ring virus. The double-stranded DNA of the viral genome was cloned, mapped (26), and sequenced (GeneBank access no. X97304). In this study we characterize for the first time a SVBV viral promoter, by infecting ZYMV-host plants with ZYMV, resulting from bombardment with ZYMV FLC under control of a putative SVBV promoter. Transient and stable expression of the reporter GUS gene in various dicotyledonous species further confirmed the functionality and applicability of the SVBV promoter for plant transformation studies.

Materials and Methods

Plant Inoculation, Maintenance and Symptom Evaluation

Squash (*Cucurbita pepo* L. cv. Ma'ayan), cucumber (*Cucumis sativus* L. cv Delila), melon (*Cucumis melo* L. cv. Arava) and watermelon (*Citrullus lanulatus* cv. Malali) plants were grown in an insect-proof greenhouse. Plants were selected for experimental use when the cotyledons had fully expanded and the first true leaf was emerging. Particle bombardment was performed using a handheld device, the HandGun (11). After bombardment, plants were grown in a closed chamber in a temperature-controlled greenhouse at 22°C. ZYMV infection was determined by ELISA and observation of symptoms 5 to 7 days post inoculation.

Construction of the Putative SVBV Promoter Upstream of ZYMV cDNA

The SVBV E-3 clone (26) served as a template to clone the putative promoter using sequence information from GeneBank (access no. X97304). A sense primer 5'GAGCGGCCGCGACCACTTGTGACTAAGGAT3' harboring a NotI site (underlined), and an antisense primer 5'GAGGCCTTCAAATGAAAATACAAGCTC 3' with a StuI site (underlined) and the transcription start codon (bold C) were employed to amplify a 950 bp putative promoter segment by PCR from the SVBV E-3 clone. The 950 bp PCR fragment was cloned into the pGEM-T vector (Promega) and termed SVBVpr in Fig. 1a.

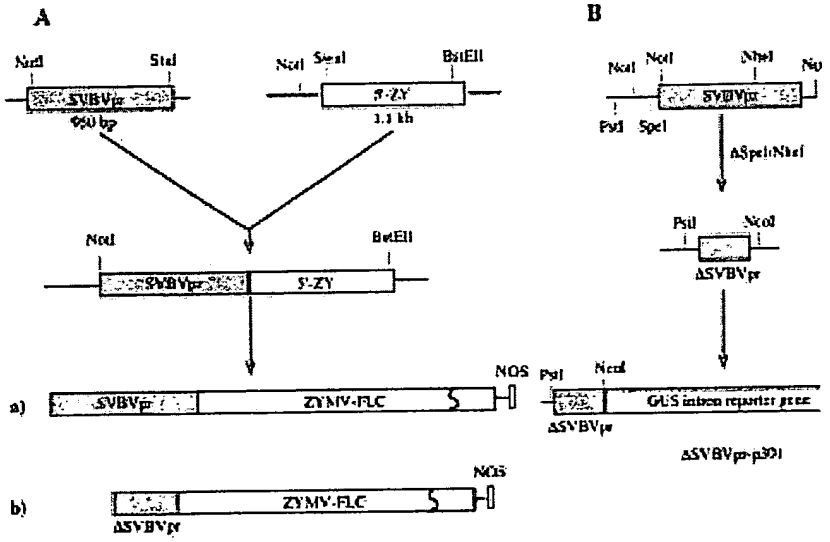

Fig. 1. Schematic drawing of the construction of the SVBV promoter (SVBVpr) upstream of the ZYMV genome (Aa) and the structure of ΔSVBV-ZYMVFLC(Ab), and (B) insertion of the ΔSVBV-promoter before the GUS reporter gene in the binary p301. The gray boxes refer to the SVBV promoter. The relevant restriction enzyme sites are marked.

A 1140 bp fragment from the 5′-region of the ZYMV full length cDNA (FLC) (6) was amplified using a sense primer 5′GCATTTAAAT AAAATTGAAACAAATCACAAAG3′ harboring a SwaI blunt-end site (underlined), and an antisense primer 5′GATCTGGGCTAGGCCTAAAC3′ which is 340 bp downstream of an unique BstEII site in the ZYMV FLC (Fig. 1A). The 1.1 kb PCR fragment produced was cloned into pGEM-T vector (Promega) and termed p5′-ZY in Fig. 1A. The SVBVpr was then linked to the 5′ non-coding region of the ZYMV-FLC. The SVBVpr fragment (NotI/StuI) was inserted into the linearized clone of p5′-ZY by excision with NotI/SwaI, generating the pSVBVpr-5′-ZY subclone (Fig. 1A). The clone pSVBVpr-5′-ZY was digested by NotI/BstEII, and the resulting fragment was used to replace a similar fragment of the NotI/BstEII segment containing the CaMV 35S promoter of the ZYMV-FLC (6) (Fig. 1A). The new FLC was designated SVBVpr-ZYMV-FLC (Fig. 1Aa).

Analysis of SVBV Promoter

The putative SVBV promoter was shortened from the 5′ end by digestion of the SVBVpr clone at unique SpeI/NheI sites and religation (Fig. 1B). This maneuver deleted a 622 bp portion of the SVBV promoter, but conserved both CCACT and TATA boxes, leaving a 328 bp fragment termed the ΔSVBV promoter (Fig. 1B). The ΔSVBV promoter was inserted into the ZYMV-FLC in the same manner as the SVBV promoter, generating ΔSVBVpr-ZYMV-FLC (Fig. 1Ab).

Construction of the Binary Vector with ΔSVBV

To examine the ΔSVBV promoter's activity following genetic transformation, CAMBIA (Canberra, Australia) binary constructs were used. The 35S promoter from plasmid pCAMBIA1p301, designated here as p301, was replaced with the ΔSVBV promoter using PstI/NcoI sites (Fig. 1B), making the ΔSVBV-p301 plasmid. The ΔSVBV-p301, the p301 (CaMV 35S promoter driving the GUS intron reporter gene), and the pCAMBIA1391Z (designated p91Z, a construct with the GUS intron reporter gene not under control of a promoter) binary plasmids were individually transformed by electroporation into *Agrobacterium tumefaciens* (strain EHA 105).

Transient and Stable Expression of GUS Driven by SVBV Promoter

After seed sterilization and germination on Murashige and Skoog (27) medium with 3% sucrose and 0.8% agar without hormones (MSO medium), seedlings of tomato (*Lycopersicum esculentum* line MP1), pepper (*Capsicum annuum* cv. Galron), cucumber cv. Bet Alpha and squash cv. Ma'ayan were grown in axenic conditions. Leaf disks cut from axenic tobacco (*Nicotiana tabacum* cv. Samsun nn) shoots grown in culture were used in transformation experiments. Overnight cultures of *Agrobacterium* in LB medium were diluted to 0.5 O.D. at 600 nm for inoculation of tobacco, tomato, cucumber and squash, or to 0.05 O.D for pepper. Tobacco and pepper explants were pre-incubated for one day on MSO medium as above, with the addition of 1 mg/l 6-benzyl adenine (BA) and 2 mg/l alpha-naphthaleneacetic acid. Cucumber cotyledon explants were pre-incubated on MSO medium, with the addition of 2 mg/l BA and 1 mg/l abscisic acid. Squash explants were incubated on MSO medium with the addition of 1 mg/l BA. Tobacco, cucumber, pepper and squash explants were dipped in *Agrobacterium* for 2–5 min. Tomato explants were inoculated as per McCormick (28). Explants were co-cultivated with *Agrobacterium* for 2–5 days, and then analyzed for β-1,3 glucuronidase (GUS) activity with a histological stain (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) (29). For evaluation of stable expression of GUS activity, tobacco explants were regenerated on selection medium (MSO containing 2 mg/l zeatin, 0.1 mg/l IAA, 20 mg/l hygromycin and 500 mg/l claforan) after 2 days of co-cultivation.

Verification of Transgenic Plants with ΔSVBV Promoter

Total genomic DNA from each transgenic line was extracted (30). Five microliters of the DNA solution were diluted in 50 µl PCR reaction mixture containing primers generated according to the published sequence of GUS (29) 5′-CAT,TAC,GCT,GCG, ATG,GAT,TCC-3′ at position 505 and SVBV promoter 5′-CAC,ATC,TTT,GCA,GCA,GAT,GTG-3′ located at position 6976. PCR conditions were: 30 cycles of 30s at 94°C, 30s at 62°C and 30 s at 72°C.

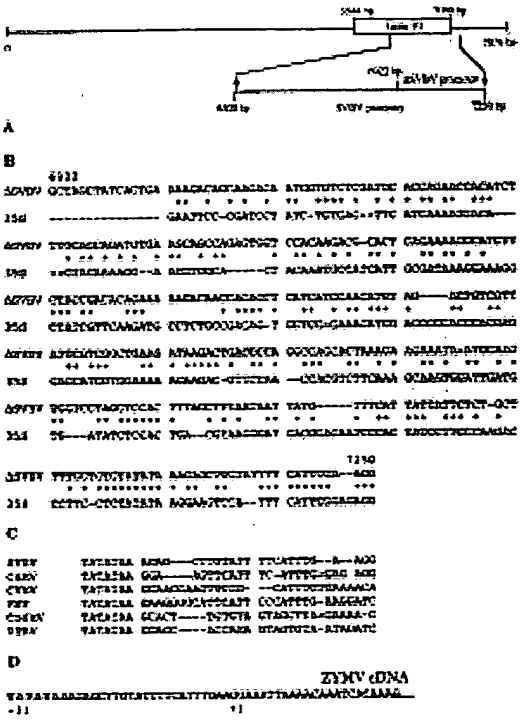

Fig. 2. Characterization and sequence comparison of the SVBV promoter. (A) ΔSVBV promoter location in SVBV genome. (B) Multiple sequence alignment of ΔSVBV promoter and 35S promoter; (−) represents gaps of alignment and (*) indicates identical nt. (C). Alignment of nucleotide sequences from the TATA box to the transcription start site of promoters from plant pararetroviruses (CaMV, CVMV, FMV, CoYMV [*Commelina* yellow mottle virus] and RTBV [rice tungro bacilliform virus]) according to (15). (D). The junction region of the SVBV promoter and the 5′-end of the ZYMV genome.

Results

Location of the SVBV Promoter

Nucleotide sequence alignment of the strawberry vein banding virus (SVBV) genome with the CaMV 35S promoter (Fig. 2b) located the CCACT box at position 7167 bp and the TATA box at position 7220 bp in the SVBV promoter. The SVBV promoter shows 61% nucleotide homology to the CaMV 35S promoter from the conserved motif CCACT to the putative transcription start site (+1) (Figs. 2B, 2C). In the 245 bp upstream of this region the homology between the viral promoters drops below 50% (Fig. 2B). The SVBV promoter mapped to start inside gene VI at positions 6300 to 7250 (Fig. 2a).

Identification of the SVBV Promoter by ZYMV cDNA Infectious Clone

The putative SVBV and ΔSVBV promoters were cloned upstream of the 5′-end of the ZYMV FLC cDNA (Figs 1Aa, 1Ab). The activities of the putative promoters SVBV and ΔSVBV were evaluated by bombarding cucurbit seedlings with DNA of SVBV-ZYMV-FLC, ΔSVBV-ZYMV FLC and 35S-ZYMV-FLC. Full infectivity was obtained with all three constructs with 10 ng/plant of DNA bombarded onto squash and cucumber, and 80–90% infectivity with melon and watermelon (Table 1). To examine the possibility of differentiation of promoter activities with our current technology different quantities of cDNAs were bombarded onto squash plants (Fig. 3).

Table 1. Transient and stable GUS expression in plant tissues transformed by binary vectors under the control of the ΔSVBV promoter, and rate of infection with SV-ZYMV cDNA

	Binary Vectors				Virus Vector
	Transient Expression			Stable Expression	Infectivity[b] Infected/Tested
Plant Species	ΔSVBV-p301	35S-p301	P91Z	ΔSVBV-p301	SV-ZYMV cDNA
Squash	+	+	−	n.t.[a]	14/14
Cucumber	+	+	−	n.t.	15/15
Tobacco	+	+	−	+ shoots[c]	
Tomato	+	+	−	+ shoots	
Pepper	+	+	−	n.t.	
Melon	n.t.	n.t.	n.t.	n.t.	13/14
Watermelon	n.t.	n.t.	n.t.	n.t.	8/10

[a]n.t., not tested. [b]Number infected out of total inoculated; bombarded with 10 ng cDNA/plant. [c]Many independent tobacco transformants were recovered.

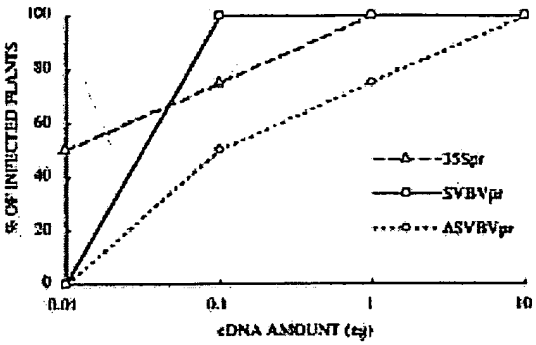

Fig. 3. Efficiency of infection of squash and cucumber plants by cloned ZYMV, under control of different promoters. Plants were bombarded with varying amounts of column-purified cDNA using the HandGun (11). Each point represents treatment with 4–5 plants.

Under control of the CaMV 35S promoter 50% ZYMV infectivity was maintained down to a cDNA dose of 0.01 ng/plant. At the same dose the SVBV-ZYMV-FLC and ΔSVBV-ZYMV FLC were no longer infective. Additionally, ΔSVBV-ZYMV FLC infectivity declined to 50% at a dose of 0.1 ng cDNA/plant, at which dose SVBV-ZYMV-FLC was still 100% infective. Symptoms were identical to the wild type virus (6).

Evaluation of the ΔSVBV Promoter by Transient and Stable Expression in Plants

Following the identification of the SVBV putative promoter by an RNA virus cDNA infectivity test, the ΔSVBV segment was inserted into a binary plasmid (Fig. 1B) to test gene expression in different plant species. Explants of tobacco, pepper, cucumber, tomato and squash were tested for transient expression of GUS after inoculation with *Agrobacterium tumefaciens* harboring the binary vectors ΔSVBV-GUS-p301, 35S-GUS-p301 (positive control), or GUS-P91Z (GUS gene without promoter). GUS expression was detected in all tested species following transformation with ΔSVBV-GUS-p301 and 35S-GUS-p301 (Table 1). The GUS histological reactions were found to be similar for these two constructs (data not shown). In all the tested species, no transient GUS expression was observed by P91Z (Table 1).

Stable expression of GUS activity under the control of the ΔSVBV promoter was observed in tobacco roots, leaves and stems of shoots regenerated

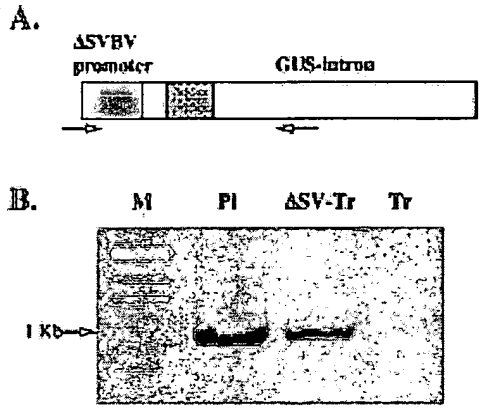

Fig. 4. Verification of the insert DNA (ΔSVBV promoter linked to GUS intron gene) within transgenic tobacco plants. A. Schematic drawing of the construct: arrows and dotted box indicate the location of the primers and the GUS intron within the construct, respectively. B. PCR analysis of a transgenic plant. DNA fragments separated by gel electrophoresis on a 1% agarose gel. The size of the predicted amplified fragment is indicated on the left. M: Lambda-Hind III/EcoRI molecular weight markers, PI is the ΔSVBV-p301 binary plasmid, ΔSV-Tr and Tr are PCR amplifications from total DNA of transformed plants with and without, respectively, the ΔSV-Gus-intron insert.

in selective conditions. A PCR fragment was generated from transgenic plants expressing a strong GUS histochemical signal, to verify that expression of the reporter gene was a result of the intact SVBV promoter (Fig. 4).

Discussion

Several TATA boxes were found within the SVBV genome (25). Comparative sequence analysis between the SVBV genome and that of CaMV located the TATA and CCACT motifs with the greatest homology. The position of the TATA box at −31 from the initiation start of transcription (Fig. 2C) is the same as that of the CaMV 35S promoter, and similar to that of analogous promoters from other viruses in the caulimovirus subgroup of the pararetrovirus supergroup (15) (Fig. 2C).

Since the development of the first plant virus infectious clone (31), many infectious clones have been produced which function after transcription *in vitro* or *in planta* (32). Transcription *in vitro* uses phage promoters, such as T3 (33), T7 (31) or SP6 (34). Transcription *in planta* until now has utilized the

CaMV 35S promoter exclusively. This is the first time, to our knowledge, that the use of a promoter other than the CaMV 35S has been reported for infection of plants with a cloned virus. Additionally in this study, a promoter was identified for first time by its action in driving a full-length clone resulting in virus infection of its host. Compared with routine procedures for promoter analysis the approach of using a viral cDNA full-length clone is rapid and easy. The sensitivity of the system is very high. Infectivity could be obtained by bombardment with small amounts of cDNA, even if the promoter is located 121 nucleotides from the 5′ end of the viral clone (6,11). The particle bombardment procedure with a viral construct, as described here, does not apparently permit analysis of the relative promoter efficiencies, as reported for stable transformation with the CaMV 35S and FMV 34S promoters linked directly to a reporter gene (35). As the level of transcription necessary to achieve virus infection is unknown, and as the viral disease is an all-or-none response, it is currently difficult to quantify promoter activity by this means. Therefore, we feel that the differences observed in Fig. 3 may not inform us about relative strengths of promoter activity (Fig. 3). The putative promoter was additionally shown to be functional in plants following transformation with *Agrobacterium*. The transient GUS expression assay in genetically transformed tissues of a variety of plant species demonstrates that the ΔSVBV promoter is also able to drive transgene transcription in several plant species, as are promoters from other caulimoviruses (15,17). Stable activity of the ΔSVBV promoter driving a reporter gene in stems, leaves and roots of transgenic plants is in a pattern similar to that reported for promoters from other caulimoviruses, such as CaMV and FMV (35). In summary, the bombardment methodology described here provides a convenient and highly sensitive approach for promoter analysis in plants.

Acknowledgments

We would like to thank Dr. Drake Stengler for the clone of SVBV, and the Israel-US Agricultural Research and Development Fund (BARD) and the Chief Scientist of the Ministry of Agriculture for grants to support this work. The Volcani Center, Bet Dagan, Israel No 548/99.

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